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Angiotensin-Converting-Enzyme Inhibitor, Lisinopril, Reduces Lipopolysaccharide-Induced Expression of Splenic Interleukin-6 mRNA in Dehydrated Rats

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Angiotensin II (ANG II) has been shown to have proinflammatory properties. To investigate whether ANG II is involved in the lipopolysaccharide (LPS)-induced production of a pyrogenic/proinflammatory cytokine, interleukin-6 (IL-6), we examined the effects of an angiotensin-converting-enzyme (ACE) inhibitor, lisinopril, on LPS-induced fever and on the expression of IL-6 mRNA in the spleen of dehydrated rats (in which the secretion of ANG II increases). The results showed that the ACE inhibitor significantly inhibited LPS-induced fever as well as the splenic expression of IL-6 mRNA in dehydrated rats. It is suggested that endogenous ANG II may be involved in the production of IL-6 that occurs in response to LPS, and thereby contribute to the LPS-induced febrile response in dehydrated rats.

Key words: angiotensin-converting-enzyme inhibitor; cytokine; interleukin-6; lipopolysaccharide; spleen

Angiotensin II (ANG II), a bioactive peptide well known to play an important role in blood-pressure and body-fluid regulation, seems to participate in inflammatory responses, too. For example, ANG II induces an inflammatory response—involving increases in the expressions of such proinflammatory enzymes as phospholipase (Schlondorff et al., 1987) and NADPH oxidase (Griendling et al., 1994)—and ANG-II-type-1 (AT₁) receptors are involved in certain types of cardiovascular inflammation (Usui et al., 2000). Furthermore, angiotensin-converting-enzyme (ACE) inhibitors have an anti-inflammatory effect (Delfraissy et al., 1984; Martin et al., 1984; Rezkalla et al., 1990). Recently, we reported results suggesting that ANG II is involved in the development of the fever (another example of an inflammatory response) induced by lipopolysaccha-

ride (LPS) in rats (Watanabe et al., 2000). In fact, the LPS-induced fever seen by us in rats was significantly attenuated by an ACE inhibitor. Since, as the first step in fever induction, the pyrogenic/proinflammatory cytokine interleukin-1 (IL-1) is released from macrophages following their stimulation with LPS (Dinarello, 1984; Kluger, 1991), we next examined the effect of the same ACE inhibitor on the fever induced by IL-1. However, this fever underwent no alterations with the ACE inhibitor (Watanabe et al., 2000), so we concluded that ANG II contributes to the LPS-induced production of IL-1 in rats.

It has repeatedly been suggested that the LPS-induced production of IL-1 in the tissues results in the local induction of IL-6 and that this then enters the general circulation to cause fever (Kluger, 1991;

Abbreviations: ACE, angiotensin-converting-enzyme; ANG II, angiotensin II; ANOVA, analysis of variance; AT₁, ANG-II-type-1; IL-1, interleukin-1; IL-6, interleukin-6; i.p., intraperitoneal(ly); i.v., intravenous(ly); LPS, lipopolysaccharide;

Luheshi et al., 1997; Luheshi, 1998). For that reason, IL-6 is now thought to be a candidate for a circulating pyrogenic cytokine (Kluger, 1991). Moreover, there is an undetectable amount of IL-1 β in the blood of animals treated with LPS (Kluger, 1991). Taking the above evidence together, it seems likely (i) that ANG II participates in the production of tissue IL-1 in response to LPS and (ii) that the local induction of IL-6 by this IL-1 might be, at least in part, involved in the development of the LPS-induced fever after the entering general circulation and acting on the brain as a circulating pyrogenic cytokine.

The present study was carried out to investigate whether the LPS-induced production of IL-6 does indeed involve an action by ANG II. To this end, the effects of an ACE inhibitor on LPS-induced fever and on the expression of IL-6 mRNA in the spleen, a representative organ of the reticulo-endothelial system, were examined in dehydrated rats. Dehydrated rats (in which the secretion of ANG II increases) were chosen, because, in our previous study (Watanabe et al., 2000), although the LPS-induced fever was significantly attenuated by an ACE inhibitor in both dehydrated and euhydrated rats, the effect was greater in the former. The present results showed that ACE inhibition significantly inhibited both LPS-induced fever and the splenic expression of IL-6 mRNA in dehydrated rats, suggesting that ANG II is involved in the LPS-induced production of IL-6 in such rats.

Materials and Methods

Animals

The animals used in this study were male Wistar rats, weighing 270–350 g. They were housed in individual plastic cages (40 × 25 × 25 cm; length × width × depth) with wood-chip bedding in a room maintained at 26 ± 1°C, a temperature within the thermoneutral zone for rats. They experienced a photoperiod of 12 h light:12 h dark, lights coming on at 0700. All animals had ad libitum access to drink and standard laboratory rat chow. The proto-

cols were reviewed by the Committee on the Ethics of Animal Experiments in Tottori University Faculty of Medicine, and the experiments were carried out in accordance with the Guidelines for Animal Experiments at Tottori University Faculty of Medicine and with the Federal Law (No. 221) and Notification (No.6) of the Japanese Government.

This study comprised 2 types of experiment (see below), both on freely moving rats. All rats were dehydrated by deprivation of drinking water for 24 h before experimentation. The rats lost about 6% of their total body weight as a result of this deprivation.

In Experiment 1, we investigated the effect of an intravenous (i.v.) injection of an ACE inhibitor, lisinopril (20 mg/kg), on the LPS (2 μ g/kg, i.v.)-induced fever in dehydrated rats. In Experiment 2, a single i.v. injection of lisinopril was given, and its effect on the LPS-induced changes in the production of IL-6 in the spleen was examined, the splenic IL-6 mRNA being measured for this purpose.

Surgery

In Experiment 1, body temperature was measured using a biotelemetry system (Lange et al., 1991; Data Science Inc., St Paul, MN). Each rat was anesthetized with intraperitoneally (i.p.) injected sodium pentobarbitone (50 mg/kg) and a battery-operated transmitter (model TA10TA-F40, Data Science) implanted i.p. The transmitter included a sensor and a radiofrequency transmitter. The output of the transmitter was monitored by antennae mounted in a receiver board (model CTR86, Data Science) placed under each animal's cage. The data were fed into a peripheral processor (matrix model BCM100, Data Science) connected to a Sanyo MBC-17J AX computer (IBM compatible) (Tokyo, Japan). The implantation of the transmitter was performed at least 1 week before the implantation of a venous cannula for i.v. injections.

Rats with i.p. biotelemetry transmitters were again anesthetized with sodium pentobarbitone (50 mg/kg, i.p.) and a polyvinyl tube was inserted into the jugular vein so that its tip lay in the superior caval vein near the right atrium (Harms and Ojeda,

1974). The free end of the catheter was passed subcutaneously to the mid-scapular region, where it was exteriorized dorsally behind the neck. It was kept patent by flushing it every day with heparinized 0.9% saline (50 U/mL). This implantation was performed at least 3 days before the start of the experiment.

In Experiment 2, the splenic expression of IL-6 mRNA was measured in dehydrated rats without transmitters. For i.v. injections, rats were catheterized as described above.

All rats were handled for 15 min each day for at least 5 days to accustom them to the experimenters.

Drugs

The LPS used in this study, which was derived from *Salmonella typhosa* endotoxin (Sigma, St. Louis, MO), was dissolved in sterile saline. Lisinopril (Sigma) was also dissolved in sterile saline. The doses injected in each experimental group are given below.

Experimental protocols

Experiment 1

Changes in body temperature were examined in conscious rats following i.v. injection of LPS (2 µg/kg). Each rat received only 1 injection of LPS, because repeated injections result in febrile tolerance. The injection of LPS was performed 30 min after an i.v. injection of either lisinopril (20 mg/kg; Lisinopril + LPS group) or saline (Saline + LPS group).

On the day of the experiment, each rat was gently picked up and its transmitter switched on using a magnet. The body temperature was then allowed to stabilize for a period of 90 min before any injections. Each injectate was given i.v. to each animal in a volume of 0.5 mL/kg over a period of 30 s. To minimize the influence of the rat's own circadian rhythm, LPS was always given between 1100 and 1200.

Experiment 2

Changes in the expression of IL-6 mRNA in the spleen were examined in dehydrated rats following an i.v. injection of LPS (2 µg/kg). Either lisinopril (20 mg/kg; Lisinopril + LPS group) or saline (Saline + LPS group) was administered i.v. 30 min before the injection of LPS. The control rats received an i.v. injection of saline (vehicle for LPS) 30 min after i.v. saline (vehicle for lisinopril) (Saline + saline group). Animals were sacrificed by CO₂ stunning followed by decapitation either 2 or 4 h after their 2nd injection (LPS or saline) and the spleen quickly removed, frozen and powdered in liquid nitrogen. This procedure was approved by the Committee on the Ethics of Animal Experiments in Tottori University Faculty of Medicine.

IL-6 mRNA: The splenic expression of IL-6 mRNA was measured by Northern blot analysis. In brief, total RNA was extracted from each tissue by the guanidinium-thiocyanate-phenol-chloroform method (ISOGEN; Nippon Gene, Toyama, Japan). The RNA (20 µg) was separated, according to size, by electrophoresis on 1% agarose gels containing 6.6% formaldehyde, transferred to a nylon membrane and subjected to hybridization. The probes were labelled with [α -³²P] dCTP by the random-priming method (BcaBEST labelling kit; Takara, Ohtsu, Japan) and purified using ProbeQuant G-50 Micro Columns (Amersham Pharmacia Biotech, Amersham, Bucks, United Kingdom). After hybridization, the membrane was washed under stringent conditions, then subjected to autoradiography. Images of the autoradiographs were taken into a personal computer and analysed [Windows, Scion Image, Plot Profile (Scion Corporation, Frederick, MD)]. The density of the IL-6 mRNA fraction, which was normalized with respect to the β -actin density in each sample, is expressed in arbitrary units.

Probes for Northern blot analysis were prepared by RT-PCR. Briefly, cDNA was made from rat spleen total RNA using an oligo(dT)₁₇ primer by means of the RT reaction. Then, PCR fragments derived from rat IL-6 and β -actin mRNA were obtained; the fragments were of 719-bp and 762-bp,

respectively. The primers used for PCR were as follows: IL-6, sense, 5'-CTTCCAGCCAGTTGC CTTCT-3' (Siegling et al., 1994), anti-sense, 5'-GCCAGTTCTTCGTAGAGAAC-3' (Bourde et al., 1996); β -actin, sense, 5'-CTATCGGCA-ATGAGC GGTTC-3', anti-sense, 5'-CTTAGGAGTTGG GGGTGGCT-3' (Siegling et al., 1994). Each PCR fragment was inserted into a T vector (pT7Blue; Novagen, Darmstadt, Germany) and cloned in *E. coli* (XL1-Blue; Stratagene, La Jolla, CA). The T vectors, which contained 1 of the above 2 PCR fragments, were selected by sequencing. Then, the T vectors were digested with *Eco*RI and *Xba*I and the probes isolated.

Statistical analysis

All results are expressed as the mean \pm SEM.

The body temperature data (Experiment 1) were analyzed for statistical significance using a repeated-measures analysis of variance (ANOVA) (Macintosh, StatView 4.0; Abacus Concepts, Inc., Berkeley, CA) to assess the overall effect. Analysis was performed on data collected from the time of drug injection onwards, i.e. from time 0.

A one-way ANOVA followed by Fisher's protected least significant difference test (post hoc test) was used to analyze tissue IL-6 mRNA data (Experiment 2).

Details of the results of the various forms of analysis are given in the figure legends. Differences were considered significant at $P < 0.05$.

Results

Effect of i.v. treatment with an ACE inhibitor, lisinopril, on the LPS-induced fever under dehydrated conditions (Experiment 1)

Figure 1 shows the effect of lisinopril (20 mg/kg, i.v.) on the LPS (2 μ g/kg, i.v.)-induced fever in dehydrated rats. A single i.v. injection of LPS induced a biphasic increase in body temperature in these rats with a latency of about 90 min. This LPS-induced fever was significantly attenuated by pre-treatment with lisinopril (Lisinopril + LPS group versus Saline + LPS group).

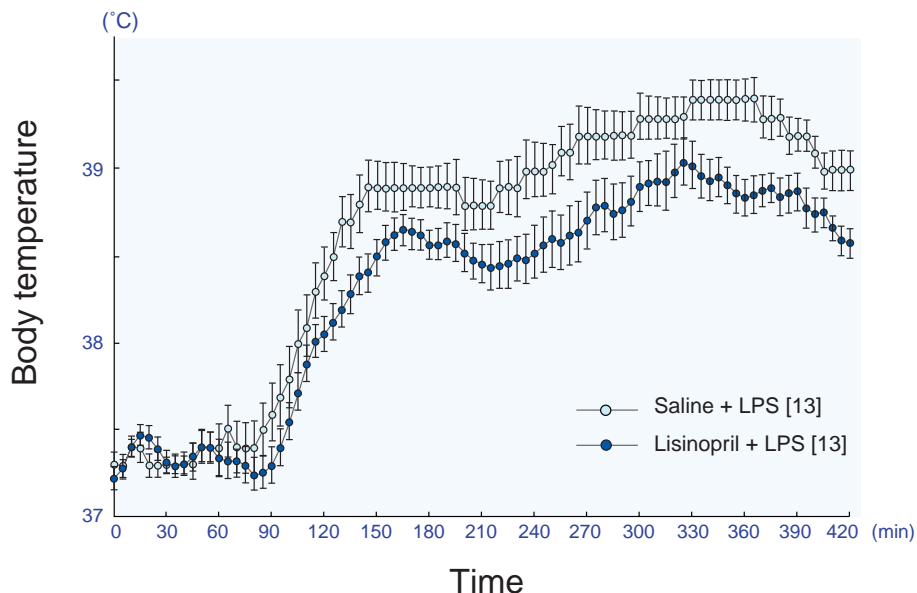


Fig. 1. Effect of an angiotensin-converting enzyme (ACE) inhibitor on lipopolysaccharide (LPS)-induced fever in dehydrated rats. Mean values (\pm SEM) obtained for body temperature ($^{\circ}$ C) in dehydrated rats after i.v. injection (at time 0) of LPS (2 μ g/kg). An ACE inhibitor, lisinopril (20 mg/kg), or saline was administered i.v. 30 min before the injection of LPS. From repeated-measures ANOVA: for treatment effect, $P < 0.05$; for time effect, $P < 0.0001$; for interaction, $P > 0.0001$. [], number of subjects.

Effect of i.v. treatment with an ACE inhibitor, lisinopril, on the LPS-induced increase in IL-6 mRNA expression in the spleen under dehydrated conditions (Experiment 2)

Figure 2 depicts the effect of a single injection of lisinopril (20 mg/kg, i.v.) on the LPS (2 µg/kg, i.v.)-induced increase in IL-6 mRNA expression in the spleen in dehydrated rats (Northern blot analysis).

As shown in Fig. 2A, LPS induced a marked increase in IL-6 mRNA expression at both 2 and 4 h after the injection (Saline + LPS group versus Saline + saline group). Pretreatment with lisinopril seemed to exert an inhibitory effect on this response at 2 h (Fig. 2A; Lisinopril + LPS group versus Saline + LPS group) and a semi-quantitative analysis of the data (Fig. 2B) revealed that the effect of lisinopril was indeed statistically significant at 2 h ($P < 0.05$).

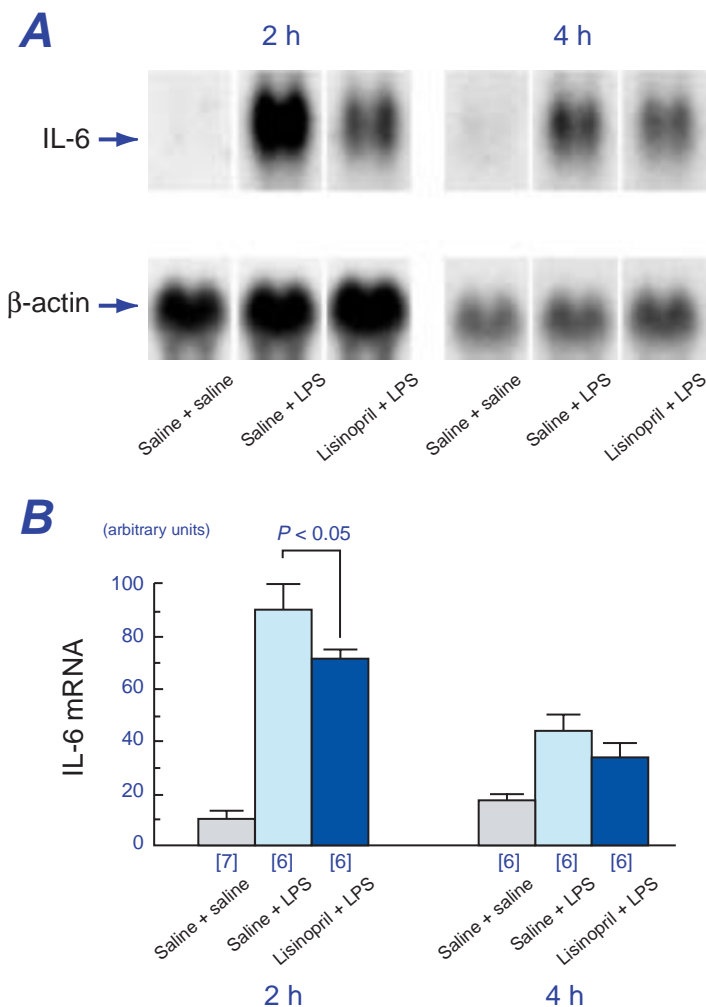


Fig. 2. Effect of an ACE inhibitor on LPS-induced increase in interleukin-6 (IL-6) mRNA expression in the spleen in dehydrated rats. IL-6 mRNA expression in the spleen of “Saline + saline,” “Saline + LPS” and “Lisinopril + LPS” groups. An ACE inhibitor, lisinopril (20 mg/kg), or saline was administered i.v. 30 min before a single i.v. injection (at time 0) of LPS (2 µg/kg) in dehydrated rats. **A:** Autoradiograms showing IL-6 mRNA and β-actin mRNA bands in a representative animal from each group. **B:** Analysis of the density of IL-6 mRNA bands in each group. Mean values (\pm SEM) obtained for IL-6 mRNA in the spleen are expressed in arbitrary units. The density of the IL-6 mRNA fraction was normalized with respect to the β-actin density for each sample. From one-way ANOVA: for treatment effect, $P < 0.0001$ (at 2 h) and $P < 0.01$ (at 4 h) in panel **B**. [], number of subjects.

Discussion

The present results showed that the LPS-induced increase in the expression of splenic IL-6 mRNA seen in dehydrated rats was significantly attenuated by treatment with an ACE inhibitor, as was the fever induced by a single i.v. injection of LPS. These results implicate ANG II in the LPS-induced production of IL-6 in dehydrated rats. Since IL-6 is thought to be a candidate for a circulating pyrogenic cytokine (Kluger, 1991), ANG II may contribute to the febrile response to LPS by mediating or modulating the LPS-induced production of IL-6. This idea is supported by our previous finding (Watanabe et al., 2000) that although this ACE inhibitor inhibits LPS-induced fever, it has no effect on IL-1-induced fever, indicating the involvement of ANG II in the LPS-induced production of IL-1 and ultimately of IL-6. At this point, it should be emphasized that IL-1 induces IL-6 in the tissues, and that the IL-6 then enters the circulation to induce part of the LPS-induced fever.

It has recently been reported that ANG II has proinflammatory properties. For example, previous *in vitro* studies have produced results that led us to speculate that ANG II contributes to the production of cytokines from leucocytes stimulated with LPS (Schindler et al., 1995; Peeters et al., 1998). Interestingly, application of ANG II onto cultured mesangial cells results in the production of IL-6 *in vitro* (Moriyama et al., 1995). Collectively, this evidence supports the present finding that ANG II is involved in the LPS-induced production of the proinflammatory cytokine IL-6 *in vivo*. However, it is unknown exactly how ANG II might contribute to the LPS-stimulated production of IL-6. One possibility is that a proinflammatory transcription factor, nuclear factor kappa-B (NF- κ B) might mediate the effect of ANG II. In fact, it has been reported that LPS activates NF- κ B in monocytes (Muller et al., 1993; Baeuerle and Henkel, 1994), that the expression of IL-6 is controlled at the transcriptional level through NF- κ B (Muller et al., 1993; Baeuerle and Henkel, 1994) and that ANG II activates NF- κ B in monocytes (Kranzhofer et al., 1999). Taken together, all this suggests the possibility that the activation of NF- κ B by LPS is enhanced by ANG II, leading to an increase in cytokine production. This idea should be tested in detail in future research.

The present study has revealed at the mRNA level that *in vivo*, ANG II is involved in the LPS-induced production of IL-6, which contributes, at least in part, to the LPS-induced febrile response in dehydrated rats. However, it is well known that ACE not only promotes the production of ANG II, but also inactivates bradykinin. Thus, ACE inhibitors increase bradykinin levels and so the elevated bradykinin could conceivably have affected our results. To strengthen our hypothesis on the role of ANG II in the production of IL-6 in response to LPS, all the experiments performed in this study should be repeated, using ANG-receptor antagonists, before reaching any final conclusions. On the other hand, we have previously shown an attenuation of LPS-induced fever by an ACE inhibitor not only in dehydrated rats but also in euhydrated rats, although the effect was greater in the former (Watanabe et al., 2000). Thus, the action of ANG II

in promoting cytokine production is not limited to dehydrated conditions. It will be interesting to investigate the significance of the stimulatory action of ANG II on the production of proinflammatory cytokines under "euhydrated conditions," using relatively high doses of LPS.

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